STOICHIOMETRY OF THE INTERACTION OF LIMA BEAN PROTEASE INHIBITOR WITH TRYPSIN AND/OR CHYMOTRYPSIN

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1. Introduction

Naturally occurring protease inhibitors are proteins which inhibit proteolytic enzymes by the formation of specific enzyme–inhibitor complexes [1]. Recently it has been observed in several laboratories [2–5] that many protease inhibitors of plant origin exist in solution as dimers, trimers and even tetramers. This aggregation phenomenon has complicated the molecular weight determination of these inhibitors and a fortiori the determination of the stoichiometry of inhibition.

Lima bean protease inhibitor (LBI) strongly inhibits both trypsin and chymotrypsin through independent combining sites [6, 7]. Based on both osmotic pressure measurements [8] and amino acid analysis [6, 9] its molecular weight is estimated at approximately 9000. However, ultracentrifugation studies [6, 8] suggest a particle size of nearly twice that value.

In this study an effort was made to establish the stoichiometry of interaction between LBI and bovine trypsin and chymotrypsin. The results clearly indicate that the monomer of LBI (M.W. $\sim$ 9000) can form 1:1 molar complexes with either enzyme and also a tertiary complex with one mole of each of these two enzymes.

2. Materials and methods

2.1. *Materials*

Lima bean protease inhibitor (LBI 8KB), trypsin (TRL OFA), and chymotrypsin (CDI-7CG) were all obtained from Worthington (Freehold, N.J.). The inhibitor was further purified by gel filtration on Sephadex G-75 [6, 9]. Malic dehydrogenase was obtained from Sigma (St. Louis, Mo) and ribonuclease A and chymotrypsinogen A were obtained from Mann (New York, N.Y.).

2.2. *Preparation of complexes*

The inhibitor (30–60 mg) was dissolved in 0.1 M NH_4HCO_3 (1–3 ml) first and then the appropriate amount of enzyme was dissolved in the inhibitor solution. After a 15 min incubation at room temperature the mixture was chromatographed on Sephadex G-75 under the conditions given in fig. 1. Appropriate fractions were combined and lyophilized.

2.3. *Inhibitor assays*

The inhibitory activities of LBI and its complexes were determined with the spectrophotometric assay previously described [7, 10]. *N*-Benzoyl-L-tyrosine ethyl ester and *p*-tosyl-L-arginine methyl ester were used as the synthetic substrates for chymotrypsin and trypsin, respectively.

2.4. *Amino acids analysis*

Samples containing 0.05–0.1 μmole of protein were hydrolyzed with 6 N HCl at 110° in sealed evacuated tubes for 22 hr. The analyses were carried out on the Spinco 120C amino acid analyzer by the method of Spackman [11] as outlined in the Spinco manual.

2.5. *Molecular weight determination*

The molecular weights of LBI and its complexes were estimated by gel filtration on Sephadex G-75 by the method described by Whitaker [12]. Samples

containing 2–10 mg of protein were dissolved in one ml of 0.1 M NH_4HCO_3 and applied to a column (1.5 X 85 cm) of Sephadex G-75 equilibrated with the same buffer. The flow rate was 11 ml/hr and 1.3 ml fractions were collected.

3. Results and discussion

3.1. *Isolation of the complexes*

Fig. 1 illustrates the isolation of the LBI-inhibitor complexes by gel filtration. In fig. 1a peak I has the properties expected for the LBI–trypsin complex (no trypsin inhibitory activity, high chymotrypsin inhibitory activity). Peak I of fig. 1b has no chymotrypsin inhibitory activity but still possesses trypsin inhibitory activity as expected for the LBI-chymotrypsin complex. Peaks II of fig. 1a and b have full inhibitory activities against both enzymes and represent excess LBI. Peak I of fig. 1c has no inhibitory activity against either trypsin or chymotrypsin and is

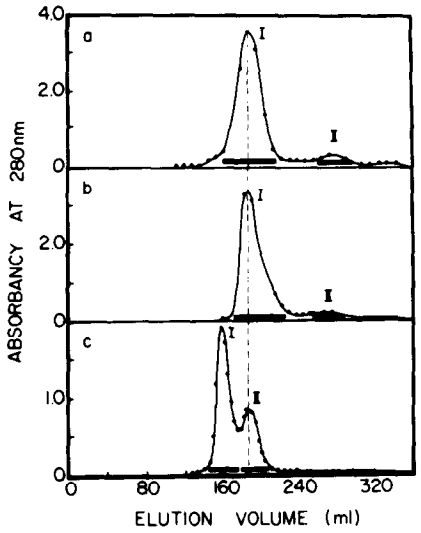

Fig. 1. Gel filtration of the complexes of LBI with trypsin and/or chymotrypsin. Columns (2.5 X 90 cm) of Sephadex G-75 were equilibrated and developed with 0.1 M NH_4HCO_3. Samples ranged from 1–3 ml; the flow rate was 22 ml/hr and 4.5 ml fractions were collected. Protein was monitored by the absorbancy at 280 nm. (a) 40 mg of LBI to which 100 mg of trypsin have been added (b) 30 mg of LBI to which 50 mg of chymotrypsin have been added (c) 50–60 mg of peak I of figure 1a to which 15 mg of chymotrypsin have been added.

therefore believed to represent the ternary complex between LBI, trypsin and chymotrypsin. Peak II of fig. 1c is believed to be excess LBI-trypsin complex (no trypsin inhibitory activity, strong chymotrypsin inhibitory activity). An elution profile similar to that of fig. 1c is also obtained when trypsin is added to an excess of peak I from fig. 1b (LBI-chymotrypsin complex).

3.2. *Molecular weight determination*

The results shown in fig. 2 indicate that LBI has an apparent molecular weight of approximately 10,000 when the sample had a concentration of 2 mg/ml. However, this value rose to ∿ 18,000, when the sample was applied at concentration of 5 mg/ml. Therefore, it appears that LBI undergoes a concentration dependent dimerization. The molecular weight of the LBI-trypsin complex was calculated to be 32,800. This is consistent with the formation of a 1:1 molar complex between enzyme and inhibitor, assuming a molecular weight of ∿ 24,000 for the enzyme and ∿ 9,000 for the inhibitor.

3.2. *Amino acid analysis*

Table 1 compares the amino composition of the LBI complexes isolated by gel filtration with the theoretical amino acid composition based on the formation of 1:1 molar complexes. The agreement, within experimental error, between experimental and theoretical values proves beyond doubt that LBI can form a 1:1 molar complex with either trypsin or chymotrypsin and can also form a ternary complex with one mole of each of these two enzymes.

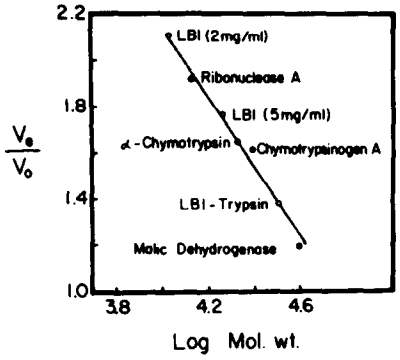

Fig. 2. Molecular weight determination of LBI and the LBI-trypsin complex by gel filtration on Sephadex G-75. (•) reference proteins; (○) sample proteins.

Table 1
Amino acid analyses of LBI, chymotrypsin (CT), trypsin (T) and the complexes of LBI with trypsin (LBI-T), chymotrypsin (LBI-CT) and with both trypsin and chymotrypsin (LBI-T-CT)[a].

Amino acid	LBI	CT	LBI-CT		T	LBI-T		LBI-T-CT	
			Theory[b]	Experiment[c]		Theory[b]	Experiment[c]	Theory[b]	Experiment[c]
Lysine	4.17	14.35	18.52	19.16	14.25	18.42	17.10	32.77	34.40
Histidine	5.37	1.95	7.32	6.52	3.42	8.79	9.90	10.74	11.05
Arginine	1.95	3.10	5.05	5.05	1.92	3.87	3.82	6.97	8.08
Aspartic acid	12.80	22.90	35.70	36.20	23.25	36.05	37.90	58.95	59.30
Threonine	5.09	23.13	28.22	29.42	11.50	16.59	16.19	39.72	38.62
Serine	11.78	26.50	38.28	39.50	34.75	46.53	47.50	73.03	73.99
Glutamic acid	6.80	15.50	22.30	23.50	14.90	21.70	22.20	37.20	37.00
Proline	5.80	10.10	15.90	15.04	9.25	15.05	15.25	25.15	23.70
Glycine	1.83	24.40	26.23	27.50	27.50	29.33	28.35	53.73	52.49
Alanine	3.18	23.30	26.38	28.50	14.82	18.00	17.10	41.20	41.60
Valine	1.30	19.55	20.85	22.80	14.60	15.90	15.74	35.45	34.40
Methionine	–	1.92	1.92	1.85	1.75	1.75	1.82	3.67	2.19
Isoleucine	3.96	7.20	11.16	11.15	13.25	17.21	18.00	24.40	25.90
Leucine	2.86	20.75	23.61	25.60	14.00	16.86	17.91	37.61	35.48
Tyrosine	1.29	4.40	5.69	5.37	11.00	12.29	11.00	16.69	14.39
Phenylalanine	1.49	6.30	7.79	9.16	2.42	3.91	4.73	10.21	10.95

[a] The analyses were carried out as described in the text. The results are expressed as moles/mole and have not been corrected for destruction of residues by acid hydrolysis.
[b] The theoretical values were obtained by adding the amino acid compositions of enzyme and inhibitor, assuming the formation of 1:1 molar complexes.
[c] The experimental values were obtained by amino acid analysis of the isolated complexes.

It is known that, when assayed with synthetic substrates, 1 mg of LBI inhibits approximately 2.5 mg of trypsin and 1.0–1.5 mg of chymotrypsin [6, 7]. The amount of trypsin inhibited is in agreement with the formation of a 1:1 molar complex, but the amount of chymotrypsin is less than expected for a 1:1 molar complex. We believe that this is due to a displacement of chymotrypsin from the LBI-chymotrypsin complex by the substrate (benzoyl-L-tyrosine ethylester). This is substantiated by the fact that in the assay system used, both LBI-chymotrypsin and the ternary complex show considerable chymotryptic activity.

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References

[1] M.Laskowski and M.Laskowski, Jr., Advan. Protein Chem. 9 (1954) 203.
[2] A.Pusztai, European J. Biochem. 5 (1968) 252.
[3] K.Hochstrasser, K.Illchman and E.Werle, Z. Physiol. Chem. 350 (1969) 655.
[4] K.Hochstrasser, K.Illchman and E.Werle, Z. Physiol. Chem. 351 (1970) 721.
[5] D.B.S.Millar, G.E.Willick, R.F.Steiner and V.Frattali, J. Biol. Chem. 244 (1969) 281.
[6] R.Haynes and R.E.Feeney, J. Biol. Chem. 242 (1967) 5378.
[7] J.Krahn and F.C.Stevens, Biochemistry 9 (1970) 2646.
[8] H.Fraenkel-Conrat, R.C.Bean, E.D.Ducay and H.S. Olcott, Arch. Biochem. Biophys. 37 (1952) 393.
[9] G.Jones, S.Moore and W.H.Stein, Biochemistry 2 (1963) 66.
[10] M.B.Rhodes, N.Bennett and R.E.Feeney, J. Biol. Chem. 235 (1960) 1686.
[11] D.H.Spackman, W.H.Stein and S.Moore, Anal. Chem. 30 (1958) 1190.
[12] J.R.Whitaker, Anal. Chem. 35 (1963) 1950.